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Production of polyclonal antibodies directed to recombinant methionyl bovine somatotropin HIGHLIGHTED

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Abstract

The administration of recombinant methionyl bovine somatotropin (rMbST) to dairy cows to increase milk yield remains a common practice in many countries including the USA, Brazil, Mexico, South Africa and Korea, whereas it has been forbidden within the European Union (EU) since 1999. A rapid screening immunoanalytical method capable of the unequivocal determination of rMbST in milk would be highly desirable in order to effectively monitor compliance with the EU-wide ban for home-made or imported dairy products. For decades, the production of specific antibodies for this recombinant isoform of bovine somatotropin (bST) has remained elusive, due to the high degree of sequence homology between both counterparts (e.g. methionine for rMbST in substitution of alanine in bST at the *N*-terminus). In this study, we compared several immunizing strategies for the production of specific polyclonal antibodies (pAbs), based on the use of the full-length recombinant protein, an rMbST *N*-terminus peptide fragment and a multiple antigen peptide (MAP) which consists of an oligomeric branching lysine core attached to the first two *N*-terminus amino acids of rMbST, methionine and phenylalanine (MF-MAP). The immunization with KLH-conjugated MF-MAP led to the production of the pAb with the highest rMbST/bST recognition ratio amongst the generated battery of antibodies. The pAb exhibited a specific binding ability to rMbST in a competitive antigen-coated ELISA format, which avidity was further improved

after purification by rMbST *N*-terminus peptide-based affinity chromatography. These results suggest that immunodiscrimination between structurally related proteins can be achieved using immuno-enhanced immunogens such as MAPs.

Keywords: recombinant methionyl bovine somatotropin, polyclonal antibodies, immunodiscrimination, multiple antigen peptide.

Abbreviations: bST, bovine somatotropin; rMbST, recombinant methionyl bovine somatotropin; MAP, multiple antigen peptide; pAb, polyclonal antibody.

1. Introduction

Bovine somatotropin (bST), with the majority isoform of 191-amino acid protein and a molecular weight of 21802 Daltons (Table 1), is produced and secreted by the anterior pituitary gland. Through a complex network system, the somatotropic axis, this hormone regulates several physiological processes involved in metabolism, growth and reproduction [1]. It is well documented that the exogenous administration of bST redirects nutritional partitioning towards milk synthesis in dairy cows, which is therefore translated into an increase in milk production ranging from 10 to 40% [2,3]. Genetically-engineered or recombinant isoforms of the bovine somatotropin have been developed and produced since the early 1990's. Recombinant methionyl bST (rMbST; Table 1), initially commercialized by Monsanto and then by Elanco (Animal Division of Eli Lilly and Company) under the trade name of Posilac®, is the only commercial product approved by the Food and Drug Administration (FDA) in the USA and by the corresponding competent authorities in Brazil, Mexico, South Africa and Korea. However, its marketing and utilization as well as the trade of dairy products obtained from rMbST-treated animals, are prohibited within the EU [4], and other countries such as Japan, China, Australia, New Zealand or Canada. Along with the on-going hormone debates between the EU and the USA, controversy has surrounded rMbST since it became commercially available in 1994, with growing concern about the implications of the administration of this synthetic protein on human and animal health and welfare. Several adverse effects reported for treated animals include diminished fertility and an increased occurrence of lameness and clinical mastitis [5, 6], which requires additional antibiotic treatments that may cause further food safety concern regarding antibiotic residues in dairy and other food products. Extensive use of antibiotics in modern agricultural farming has also been linked to the development and emergence of antibiotic resistance that is currently affecting both human and veterinary medicine worldwide [7]. Moreover,

administration of somatotropin raises the concentration of Insulin-like Growth Factor 1 (IGF-1) in milk [8]. Elevated circulating levels of IGF-1 have been associated with a higher risk of developing several types of cancer [9, 10]. However, studies correlating the intake of milk from rMbST-treated animals with human diseases are still lacking.

In order to control illegal administration of rMbST and to ensure high quality and safety of milk and consumer protection, reliable analytical methodologies capable of unambiguous identification of the synthetic methionyl growth hormone in milk are required. Current analytical methods for determination of rMbST rely on instrumental technologies such as HPLC-MS/MS [11-14]. Despite the fact that chromatographic systems are highly sensitive and specific, these techniques often limit their applications for rapid screening of a large number of samples due to the requirement of extensive sample preparation time and sophisticated instrumentation which is also laboratory-based. Immunoanalytical methods, in particular Enzyme-Linked Immunosorbent Assays (ELISA), are widely used as rapid screening tools for routine monitoring of food contaminants and residues, owing to their simplicity, cost-effectiveness and capabilities of performing high-throughput analysis. Two different immunoanalytical approaches have been adopted for the detection of rMbST. By the direct strategy, the presence of the native and the recombinant isoforms is determined simultaneously in biological fluids [15-17], whereas the indirect approach is based on the analysis of biomarkers of which their concentration is increased upon rMbST administration. IGF-1 has been the traditional target measured for this purpose [8, 17-20]. Methods based on the detection of anti-rMbST immunoglobulins in treated cows have also been published [21, 22]. Nevertheless, the direct analysis of rMbST itself is highly preferable, in order to circumvent problems associated with inter and intra-individual variation of biomarkers expression levels, which can lead to misinterpretation of results.

106 To date, no specific immunoassays have been described for the detection of rMbST. The high
107 sequence homology displayed by the recombinant and the native somatotropins (methionine
108 in substitution of alanine at the *N*-terminus) has greatly hindered the successful production of
109 rMbST-selective antibodies. The strategy most often used entails the immunization with the
110 complete recombinant protein [21, 23-25]. Nevertheless, the antibodies produced following
111 this procedure have generally failed in their capacity to immunodiscriminate between bST
112 and rMbST, while a 2–fold increased affinity factor towards rMbST was described for the
113 mAb-based sandwich assay developed by Erhard *et al* [23]. Considering that only one amino
114 acid of difference at the *N*-terminus is encountered, the immunization with the whole protein
115 most likely leads to the production of antibodies directed towards shared epitopes in both
116 counterparts, bST and rMbST, therefore being unable to specifically recognize the latter. On
117 the other hand, a frequently accomplished practice for raising antibodies against proteins is
118 based on the use of immunizing synthetic peptide fragments which mimic concrete sequences
119 within the target [26-28]. The immunization with a synthetic peptide representing the
120 differential *N*-terminus of rMbST could *a priori* focus the immune response towards the
121 recognition of the characteristic epitope of the protein. Castigliego *et al* described for the first
122 time the production of a mAb by using a synthetic nine amino acid rMbST *N*-terminus-
123 mimicking peptide coupled to KLH as immunogen [16]. Despite showing a 3–fold higher
124 affinity towards rMbST than to bST, complete immunodiscrimination was not yet possible by
125 using the developed immunoassays. As an alternative to monovalent peptides, multiple
126 antigen peptides (MAPs) or multimerized peptides, have been used as immunogens since
127 they were developed in 1988 [29], especially in the area of vaccine development [30-32].
128 MAPs have been shown to efficiently improve the immunogenicity of a particular antigen,
129 thus eliciting a stronger immune response, as a consequence of the presentation of multiple
130 copies to the immune system [33]. Furthermore, it has been reported that the resulting

immune response is generally mono-specific and more homogeneous [34]. The production of pAbs targeted at vertebrate somatotropins using a synthetic MAP constituted by several copies of an 18 amino acid highly conserved domain proximal to the *C*-terminus of the protein has already been published [35]. To our knowledge this system has never been applied before for the production of rMbST-specific antibodies.

In the present work several immunization strategies have been compared for the production of anti-rMbST rabbit pAbs. The immunization with an octavalent synthetic rMbST *N*-terminus dipeptide-mimicking MAP, followed by affinity purification with a synthetic rMbST *N*-terminus-mimicking linear peptide, resulted in the production of a pAb capable of specifically differentiating between the recombinant and the native somatotropins in a competitive ELISA format.

2. Experimental

2.1. Reagents and instrumentation

3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 2-(*N*-morpholino)ethanesulfonic acid, 2-(4-morpholino)ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Aldrich (St. Louis, MO, USA). Succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) was acquired from Thermo Fisher Scientific (Rockford, IL, USA). Slide-A-Lyzer dialysis cassettes and Zeba 7kDa Desalting columns from Thermo Fisher Scientific (Rockford, IL, USA) were used for the purification of the immunizing conjugates. Keyhole limpet hemocyanin (KLH) carrier protein, complete and incomplete Freund's Adjuvants, the bicinchoninic acid (BCA) test kit were from Sigma Aldrich (St. Louis, MO, USA). rMbST *N*-terminus dipeptide-mimicking MAP, MF-MAP-C (Figure 1), was acquired from GenScript

(Piscataway, NJ, USA). Synthetic rMbST *N*-terminus-mimicking linear peptides EP091213 (amino acid sequence: H_2N–Met-Phe-Pro-Ala-Met-Ser-Leu-Ser-Gly-Leu-Phe-Ala-Asn-Ala-Val-Leu-Arg-Cys–COOH) and EP093536 (amino acid sequence: H_2N–Met-Phe-Pro-Ala-Met-Ser-Leu-Ser-Gly-Leu-Phe-Cys–$CONH_2$), used for animal immunization and antibody purification, respectively, were purchased from Eurogentec S.A. (Seraing, Belgium). Affinity columns packed with Protein A Sepharose 4 Fast Flow gel purchased from GE Healthcare were used for the purification of rabbit antibodies. EP093536 peptide affinity column was prepared using Toyopearl AF-Amino-650M gel from Tosoh Bioscience GmbH (Stuttgart, Germany). Maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) and streptavidin poly−HRP (STV−pHRP) were acquired from Thermo Fisher Scientific (Rockford, IL, USA). Centricon Plus-20 ultracentrifugation concentration devices (molecular cut-off 3K) were purchased from Millipore (Billerica, MA, USA). Peroxidase-conjugated polyclonal goat anti-rabbit immunoglobulin (GAR−HRP) was purchased from Sigma Aldrich. Biotin-SP-conjugated polyclonal goat anti-rabbit IgG (H+L) (GAR−b) was obtained from Jackson Immunoresearch Europe (Suffolk, United Kingdom). Gelatin was from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) was from Sigma Aldrich. Lactoferrin from bovine milk was provided by Taradon Laboratory SPRL (Tubize, Belgium). β-lactoglobulin from bovine milk was from Sigma Aldrich. Casein from bovine milk was purchased from Merck. Bovine prolactin was from the National Institute of Health (NIH, USA). Bovine placental lactogen was provided by Jean-François Beckers from the University of Liège (Liège, Belgium). Pepstatin, EDTA and acetic acid were from Sigma Aldrich (St. Louis, MO, USA). Sequencing-grade modified trypsin (EC 3.4.21.4) was from Promega (Madison, WI, USA).

Ninety-six-well flat-bottom Nunc Maxisorp polystyrene ELISA plates were purchased from Nunc (Roskilde, Denmark). ELISA plate washer model 1575 Immunowash was from Bio-Rad Laboratories (Hercules, CA, USA). 3,3',5,5'-Tetramethylbenzidine/H_2O_2 solution was

179 from BioFX Laboratories (Owings Mills, MD, USA). ELISA absorbance was monitored at
180 450 nm using a Multiskan EX reader provided by Thermo Fisher Scientific (Zellik, Belgium).
181 Buffers and solutions: *1) Coating buffer (CB)*: 50 mM sodium carbonate–bicarbonate buffer
182 (pH 9.6). *2) Blocking solution*: CB containing 0.5% (w/v) gelatin. *3) EIA buffer*: 5.6 mM
183 $Na_2HPO_4 \cdot 2H_2O$, 0.9 mM $NaH_2PO_4 \cdot 2H_2O$, 150 mM NaCl, (pH 7.4), containing 0.02% (w/v)
184 gelatin, 0.005% (v/v) Tween 20, 0.001% (w/v) 8-Anilino-1-naphthalenesulfonic acid
185 ammonium salt, 0.005% (w/v) ascorbic acid, 0.01% (w/v) thimerosal. *4) Washing solution*:
186 150 mM NaCl, containing 0.05% (v/v) Tween 20. *5) PBS*: 44 mM sodium phosphate, 6 mM
187 potassium phosphate, 154 mM NaCl, (pH 7.4). *6) PBST*: PBS containing 0.05% (v/v) Tween
188 20.

189 **2.2. Somatotropin standards**

190 Pituitary bovine somatotropin (bST) was purchased from the National Hormone and Peptide
191 Program (NHPP), Harbor-UCLA Medical Centre (Torrance, CA, USA). Pituitary porcine
192 somatotropin (pST) was from Sigma Aldrich. Recombinant methionyl equine somatotropin
193 (rMeST, EquiGen-5) was from Bresagen Ltd. (Thebarton, Australia). Recombinant methionyl
194 bovine somatotropin (rMbST) was extracted from the slow release formula of Lactotropin®
195 syringes provided by Elanco (Greenfield, IN, USA) as described previously with
196 modifications [25]. Briefly, 20 mL of 50 mM CAPS buffer (pH 11.0; 100 mM NaCl) was
197 added to 500 mg of syringe content. The syringe content was emulsified by vortex for 1
198 minute and then by sonication for 10 minutes in a water bath. The emulsion was immediately
199 centrifuged (1000×g, 10 min) and the transparent layer containing rMbST was removed from
200 the white excipient layer. To ensure maximum recovery of rMbST a further 10 mL of CAPS
201 buffer was emulsified with the remaining white excipient and mixed by vortex and sonication
202 and centrifuged as previously described. The rMbST phases were pooled together and filtered

using a 0.45 µm cellulose filter. The rMbST solution was immediately aliquoted, lyophilized and stored at –20 °C until use. The protein concentration of the extracted rMbST was determined using a BCA assay and confirmed by liquid chromatography coupled to high-resolution mass spectrometry measurement (LC-HRMS^n; results not shown), as described previously [11]. A tryptic digestion was performed overnight at 37 °C in 120 µL of 50 mM ammonium bicarbonate, 10 mM EDTA and 1 µM pepstatin (pH 7.9) with 2 µg of enzyme. The extracts were then evaporated, reconstituted in 40 µL of water/acetonitrile 70:30 (v/v) with 0.2% (v/v) formic acid and analyzed by LC-HRMS^n. Separation of the peptides was achieved on a Symmetry 300 2.1 mm × 150 mm C4 column packed with 3.5 µm beads, 300 Å pore size (Waters, Milford, USA) . The solvent flow rate was set at 300 µL min^{-1}. Peptides were separated using acetonitrile containing 0.2% (v/v) formic acid (A) and water containing 0.2% (v/v) formic acid (B) as mobile phase. The elution gradient started with 10% A increasing to 50% in 5 min, then decreasing to initial conditions in 5 min and remaining at 10% A for 5 more minutes. A divert valve was used to let the sample pass into the instrument from 4.5 to 9 min. The typical expected retention time was 6.7 and 6.9 min for the rMbST *N*-terminus peptide and the rMeST *N*-terminus peptide (used as internal standard), respectively. The MS instrument was a linear ion trap coupled to an orbitrap allowing high resolution measurements (LTQ-Orbitrap^{TM}, Thermo Electron, Bremen, Germany), fitted with an electrospray ion source (ESI). The API interface was operated in positive ion mode. A sample volume of 20 µL was loaded onto the column using the autosampler. A column heater was used to ensure a stable column temperature of 30 °C. Mass spectrometric analyses were performed in the following working conditions: capillary voltage was set at 42 V, source voltage at 5 kV and capillary temperature at 300 °C. Nitrogen was used as sheath, auxiliary and sweep gas at flow rates of 50, 10 and 10 (arbitrary unit), respectively. The linear ion trap mass spectrometer was set to select the ions 933.5 and 913.3 corresponding to $[M+2H]^{2+}$ of

the tryptic *N*-terminus peptides of rMeST and rMbST, respectively. Collision energy of 20% (arbitrary unit) was applied to the ion 933.5 and 18% to the ion 913.3. The detection of the resulting product ions was performed in the orbitrap at a resolution of 30 000. Acquisition was performed in full scan mode from m/z 500 to 1500. Data were collected and analyzed with the Xcalibur software (Thermo Electron).

2.3. Preparation of immunogens

i) Coupling of rMbST to KLH: the immunogen rMbST–KLH was obtained using the EDC/NHS reaction. Briefly, 20 mg of EDC and 10 mg of NHS were dissolved in 100 µL of 0.05 M MES, 0.5 M NaCl buffer (pH 4.7). The solution of EDC/NHS was added to 10 mg of rMbST and allowed to react for 10 min. The EDC/NHS-activated rMbST was added slowly with stirring to 1 mL of PBS (pH 7.4) containing 20 mg of the carrier protein. The conjugation reaction was performed overnight under stirring at room temperature.

ii) Coupling of cysteine-containing rMbST N-terminus peptides to KLH: synthetic rMbST *N*-terminus-mimicking peptides, EP091213 and MF-MAP-C (Figure 1), were conjugated to KLH via their cysteine residues using the commercial sulfo-SMCC conjugation kit (Fisher Scientific UK, Leicestershire, UK) according to manufacture instructions.

All of the immunogens were purified by overnight dialysis (Slide-A-Lyzer Dialysis cassettes; 3kDa cut-off) against a 0.9% (w/v) NaCl solution. The protein concentration was estimated by BCA assay and the conjugates were aliquoted, lyophilized and stored at −20 °C.

2.4. Polyclonal antibody production

Polyclonal antibodies were produced in New Zealand white SPF (specific pathogen free) rabbits. A summary of the production of the pAbs is shown in Table 2. Rabbits were immunized by subcutaneous injection with 1 mL of a 1:1 emulsion of a saline solution containing the immunogen and Freund's complete adjuvant (first dose) or Freund's

incomplete adjuvant (subsequent doses). Generally 0.2 mg of immunogen were administered, with the exception of rMbST–KLH, for which 0.1 mg were used. Immunizations were performed initially in intervals of 15 days, and then monthly after the third boost. A test blood sample was obtained from every rabbit prior to immunization, and then 10 days after each immunization from the 3rd boost onward. Pre-immune and immune sera were obtained by coagulation and centrifugation of the blood samples. Working solutions were prepared in a 1:1 mixture of EIA buffer and ethylene glycol, and they were stored at –20 °C.

2.5. Peptide-based affinity purification of the polyclonal antibody MU11

The pAb MU11 was purified by affinity chromatography using a peptide EP093536 column.

i) Peptide EP093536 affinity column preparation: the $-NH_2$ groups of the Toyopearl AF-Amino-650M gel were activated with MBS, followed by the coupling of the maleimide activated $-NH_2$ moieties with the –SH group of the cysteine in the peptide EP093536. 2.5 mL of gel in 1.25 mL of 50 mM sodium phosphate buffer (pH 6) were incubated for 30 min at room temperature with orbital agitation with 175 µL of a MBS solution in *N*,*N*-dimethylformamide (DMF; 15 mg/mL). The gel suspension was then centrifuged (1800×g, 2 min) and supernatant was discarded. The gel was washed 3 times with 2.5 mL of 10× PBS (pH 7.4), and resuspended in 1.25 mL of PBS. Immediately prior to use, the peptide EP093536 (8.4 mg) was dissolved in 1.4 mL of a mixture of DMF:PBS (30:70, v/v). The peptide solution was added to the gel suspension and incubated for 18 h at room temperature under orbital agitation. After centrifugation, the gel was resuspended in 2.5 mL of PBS and packed into a purification column. The peptide EP093536 affinity column was stored at 4 °C.

ii) Purification of the pAb MU11 by peptide-based affinity chromatography: prior to the peptide-based purification, the immunoglobulin fraction of the rabbit pAb MU11 was isolated from the crude serum (5 mL) by protein A affinity chromatography. The recovered

immunoglobulin fractions were then dialyzed in PBS (18 h, 4 °C), and concentrated in a Centricon Plus-20 device (4600×g, 30 min, 4 °C) up to a final volume of 1 mL. The solution containing the immunoglobulins was loaded onto the peptide EP093536 column pre-conditioned by the addition of 5 volumes of PBS (20 g/L NaCl), and incubated at room temperature for 3 h with orbital agitation. The non-retained immunoglobulins were eluted by washing with PBS (20 g/L NaCl). The elution of the peptide-specific immunoglobulins was conducted using 100 mM glycine buffer (pH 2.5). The column was regenerated by addition of 5 volumes of PBS and stored at 4 °C in 20:80 methanol:distilled water. Those fractions containing immunoglobulins, as determined spectrophotometrically at 280 nm, were pooled and dialyzed overnight in PBS containing 0.001% (w/v) NaN_3 at 4 °C. The antibody solutions (specific and non-specific fractions) were concentrated as described above, and the concentration was estimated by the BCA protein assay test. The antibodies were stored at –20 °C in PBS containing 0.001% (w/v) of thimerosal and 10% (w/v) of BSA.

2.6. Antigen-coated competitive ELISA

i) *General assay procedure:* ELISA plates were coated overnight at room temperature with 100 µL of the standard solution [rMbST or bST] prepared in coating buffer (ranging from 0.25 to 16 µg/mL). The blocking of the plates was performed by incubation for 2 h at 37 °C with 250 µL of blocking solution. After the coating and the saturation steps, the plates were aspirated. In the competitive assay, 50 µL of the antibody solution and 50 µL of the standards prepared in EIA buffer were added and incubated for 1 h at 37 °C. Then, 100 µL of labeled secondary antibody prepared in EIA buffer (GAR–HRP at 1:2000) was added and incubated for 1 h at 37 °C. Plates were washed 5 times with washing solution between each incubation step. Finally, the retained peroxidase activity was revealed with 100 µL of a

299 ready-to-use TMB solution for 30 min in the darkness at room temperature. The enzymatic
300 reaction was stopped by addition of 50 µL of 1.8 N H_2SO_4.
301 *ii)* *Optimized assay procedure:* the following procedure was used for the purified pAb
302 MU11. This protocol was based on the general assay procedure, with slight modifications. An
303 overnight pre-incubation of the antibody and the antigen (mixed at equal volumes in plastic
304 tubes) was performed at 4 °C. Then, 100 µL of the pre-incubated solutions were added to the
305 coated and blocked plates, and incubated for 30 min at 37 °C. A biotin/streptavidin
306 amplification system was used, consisting of an initial incubation with 100 µL of a 1:150000
307 dilution of GAR–b prepared in PBST (1 h, 37 °C), followed by an additional incubation with
308 100 µL of a 1:20000 dilution of STV–pHRP prepared in PBST containing 1% (w/v) of BSA
309 (1 h, 37 °C).
310 *iii)* *Signal processing:* Absorbance was monitored at 450 nm. The signal intensity was
311 plotted against the standard concentration in a logarithmic scale, and the resulting sigmoidal
312 curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot
313 software package from SPSS Inc. (Chicago, IL, USA). The IC_{50} value, corresponding to the
314 standard concentration that generates a 50% reduction of the maximum signal intensity
315 (A_{max}), was used for the estimation of the assay detectability. The limit of detection of the
316 assay (LOD) was determined as the concentration of standard that generates a 2sd decrease of
317 the signal obtained at the zero dose of analyte (A_0 – 2sd). The absorbance values were
318 normalized using A_0 as reference measure.

319 **2.7. Cross-reactivity study**

320 The capability of recognition by the peptide-purified antibody MU11 to pituitary bovine
321 somatotropin (bST), pituitary porcine somatotropin (pST), recombinant methionyl equine
322 somatotropin (rMeST), bovine serum albumin (BSA), lactoferrin, β-lactoglobulin, casein,

bovine prolactin and bovine placental lactogen was evaluated by conducting competitive experiments using the mentioned proteins as competitors. Cross-reactivity (CR) values were calculated as follows: $CR = IC_{50(rMbST)}/IC_{50(competitor)} \times 100$.

3. Results and discussion

3.1. Immunogen description

In the present work several immunizing strategies for the production of anti-rMbST antibodies were compared. The immunogens were categorized as i) complete recombinant somatotropin (rMbST); ii) rMbST *N*-terminus-mimicking synthetic linear peptide; and iii) rMbST *N*-terminus-mimicking synthetic MAP. In the case of the synthetic molecules (linear peptide and MAP), the design comprised a *C*-terminus cysteine, an amino acid commonly introduced for coupling purposes, via the side chain thiol group. The rMbST-mimicking synthetic linear peptide EP091213 represents the first 17 *N*-terminus amino acids of rMbST (H_2N–MFPAMSLSGLFANAVLRC–COOH). The rMbST-mimicking synthetic multiple antigen peptide MF-MAP-C (Figure 1), displays in an *arachnid*-type manner eight units of the first two *N*-terminus amino acids of rMbST, methionine and phenylalanine, with a weakly immunogenic $lysine_n$ core. All of the immunogens were prepared by covalent conjugation of the three mentioned molecules to the carrier protein KLH. In order to guarantee the accessibility of the rMbST *N*-terminus characteristic portion of the antigens to the immune system, different coupling chemistries were used for the preparation of the immunogens. In the case of the recombinant whole protein, formation of amide bonds between the free carboxylic groups of rMbST and the amine groups of KLH was conducted using the active ester method. The peptide EP091213 and MF-MAP-C were conjugated using the

heterobifunctional crosslinker sulfo-SMCC, by coupling each peptide to maleimide-activated amine groups of KLH via the cysteine residue of their *C*-terminus.

By the immunization of rabbits with the three mentioned immunogens, 9 polyclonal antibodies were obtained (Table 2).

3.2. Preliminary antibody characterization

The capability of immunodiscrimination of the 9 available pAbs between the native and the recombinant methionyl somatotropins was initially assessed by a checkerboard titration procedure in the antigen-coated ELISA format using both proteins as coating antigens. As a first approach, the crude sera were analyzed without any further purification. As shown in Table 3, all three immunogens gave rise to a positive immune response. All of the antibodies, with only one exception (M10), recognized the native somatotropin and/or the recombinant methionyl isoform to a different extent. Despite immunization with rMbST–KLH generated the pAbs with the highest titers, these were unable to immunodiscriminate the recombinant methionyl isoform, therefore displaying a very similar binding behavior towards both somatotropins. From these results and those described in previous studies where the same immunizing strategy was used [21, 23-25], it could be inferred that using the complete recombinant protein as immunogen directs the immune response towards common antigenic determinants within the recombinant and the native isoforms, with the rMbST *N*-terminus being "masked". This remark is of special relevance in the case of pAbs, representing a heterogeneous collection of antibodies with disparate profiles of selectivity, where the rMbST-specific sub-population, if produced, would consequently be in the minority. Finally, the immunogens consisting of rMbST *N*-terminus-mimicking synthetic peptides (both the linear peptide and the MAP), afforded the most promising results, as three of the produced pAbs exhibited a higher recognition towards rMbST, namely M9 for EP091213–KLH, and

MU9 and MU11 for MF-MAP-SMCC–KLH. These antibodies were therefore selected for further studies.

In order to reduce background-associated problems encountered with the raw sera, the three pAbs were purified by protein A affinity chromatography. A representative set of data of the response to rMbST and bST of the mentioned protein A purified antibodies by checker-board titration in the antigen-coated ELISA is displayed in Figure 2, including also one of the generic pAbs (BC8) for comparative purposes. The highest rMbST/bST recognition ratio was observed for pAb MU11, therefore highlighting the superior efficiency of the immunogen based on the rMbST *N*-terminus-mimicking MAP coupled to KLH over the other strategies used. Despite the fact that suitable amino acid number in MAPs is usually considered to be comprised between 10 and 20 residues [35], the immunization with an rMbST *N*-terminus dipeptide-mimicking MAP has been proven to be sufficiently immunogenic to induce the production of anti-rMbST antibodies.

In conclusion, as previously reported by other authors regarding the production of antibodies directed to other targets [33, 36], the immunization with a MAP resulted in a stronger and/or more specific response than that generated by a mimicking monovalent peptide or the complete recombinant protein. According to the results herein presented, the rabbit pAb MU11 was chosen for the development of an anti-rMbST immunoassay.

3.3. Competitive immunoassay based on the polyclonal antibody MU11

Those combinations of antibody dilution/coating antigen (rMbST) affording adequate signal intensity (around 1 absorbance units), as determined by checker-board titration, were selected to perform inhibition experiments using rMbST and bST as competitors. A preliminary experiment was carried out to determine the assay conditions generating the highest inhibition ratios for rMbST. It was found that the introduction of a pre-incubation step of the

antibody and the competitor in combination with a short period of time for the competitive step provided an improvement of the assay sensitivity (results not shown). Compensation of signal loss due to the reduction of the immunoreactive step time was achieved using a signal amplification system based on a biotinylated secondary antibody and HRP–labeled streptavidin. As shown in Figure 3, a specific response was displayed by the antibody MU11 towards rMbST, whereas no inhibition was observed when bST was used as competitor. All of the evaluated combinations afforded very similar inhibition ratios for rMbST, with an estimated IC_{50} value comprised between 500 and 5000 μg L^{-1}. In order to improve the antibody performance in terms of sensitivity, the pAb was subjected to a further purification step using an rMbST *N*-terminus-mimicking synthetic peptide-based affinity procedure. The affinity purification of antibodies produced against rMbST has been previously reported, although in all of the described methods the complete recombinant protein was used with this purpose [16, 17, 21, 25]. The competitive standard curves for rMbST and bST, as well as the assay conditions and parameters obtained using the peptide-purified MU11 pAb in the most sensitive coating antigen/antibody dilution combination are included in Figure 4. By using the peptide-purified fraction of the pAb MU11 the assay sensitivity was greatly improved, being the resulting LOD for rMbST in buffer of 66 μg L^{-1}. This result indicates that antigen-based affinity purification is a convenient strategy not only to isolate the immunoglobulin sub-populations directed to a ligand, but also to modulate the overall avidity of a pAb, and thus the assay sensitivity, as those immunoglobulins with the lowest affinity towards the target are removed during the washing step. Levels of rMbST/bST in fresh bovine milk after administration of somatotropin slow release formulations have been reported to be below 5 μg L^{-1} [15, 37], in contrast to plasma or serum, where concentrations up to 120 μg L^{-1} have been found [13]. Furthermore, common heat treatments to which commercial milk is submitted prior to commercialization, such as pasteurization, reduce up to 90% the

418 rMbST/bST content [14, 37]. In order to effectively monitor the presence of rMbST in milk
419 samples, further improvement of the MU11-based immunoassay herein presented is therefore
420 required, or alternatively, the production of additional receptors which display the specificity
421 to rMbST shown by pAb MU11, altogether with an increased affinity towards the target. As a
422 first approach, immunization with rMbST-mimicking MAPs bearing longer peptides will be
423 attempted, in order to determine the influence of the length of the displayed subunits in the
424 MAP on the avidity of the produced antibodies.

425 **3.4. Cross-reactivity analysis**

426 The interaction of the peptide-purified MU11 pAb with bST and somatotropins from other
427 species (either native or recombinant; pST and rMeST), BSA, milk bovine proteins
428 (lactoferrin, β-lactoglobulin and casein), as well as bovine proteins displaying a high
429 sequence homology with bST (lactogen and prolactin) was assessed in a cross-reactivity
430 study, using the mentioned molecules as competitors. No recognition was observed to any of
431 the evaluated proteins, with the exception of rMeST, for which a CR value of 5.6% was
432 obtained. The pituitary somatotropins which were not recognized by MU11, bST and pST,
433 lack the *N*-terminus methionine present in the dipeptide displayed by the immunogen used for
434 the production of the antibody (MF-MAP-SMCC–KLH), finding which points out the crucial
435 role of this residue as antigenic determinant in the antibody-antigen binding event.
436 Comparing the *N*-terminus amino acid sequence of rMbST (H_2N–
437 MFPAM**S**LS**G**LFANAVLRA–) with that of rMeST (H_2N–MFPAM**P**LS**S**LFANAVLRA–),
438 whereas both recombinant proteins present the *N*-terminus methionine, two differences are
439 encountered (highlighted in bold). Given the decreased interaction observed for rMeST with
440 respect to rMbST, serine at position 6 and/or glycine at position 9 appear to be also required
441 for the antibody recognition. Presumably, the additional purification of the antibody using the

rMbST *N*-terminus-mimicking linear peptide, would have contributed to the selection of immunoglobulins which paratope would better fit an epitope containing these two amino acids, therefore being specific for rMbST.

4. Conclusions

For decades the production of specific antibodies directed towards rMbST has not been successfully accomplished as a consequence of an extremely minor difference (one amino acid) encountered at the *N*-terminus of both proteins. In this paper, we report the generation of a rabbit pAb displaying a high selectivity towards rMbST in a competitive antigen-coated ELISA format. The immunogen employed for the production of the pAb consisted in an rMbST *N*-terminus-mimicking synthetic MAP displaying the first two amino acids of rMbST conjugated to the carrier protein KLH. Further purification of the antibody using an rMbST *N*-terminus-mimicking synthetic linear peptide significantly improved the performance of the antibody. Further work is currently ongoing in order to produce rMbST-specific monoclonal and polyclonal antibodies to efficiently develop an immunoassay that meets the requirements both in terms of specificity and sensitivity to be implemented for the routine screening of rMbST in milk.

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529

529 **Table and Figure captions**

530 **Table 1**. Pituitary and commercial recombinant bST specifications.

531 **Table 2**. Polyclonal antibody production.

532 **Table 3**. Summary of the preliminary characterization of the pAbs by antigen-coated ELISA.

533 **Figure 1**. Chemical structure of the rMbST *N*-terminus-mimicking immunizing synthetic
534 multiple antigen peptide (MAP).

535 **Figure 2**. Recognition towards rMbST and bST coating antigens (4 μg mL^{-1}) displayed by
536 different pAbs.

537 **Figure 3**. Competitive experiment performed with the protein A purified pAb MU11 using
538 bST and rMbST as competitor reagents (empty and filled bars, respectively). rMbST coating
539 antigen concentrations were 1 (■), 2 (■), 4 (■), 8 (■) and 16 (■) μg/mL, and they were
540 combined with the following antibody dilutions: 1/500, 1/500, 1/1000, 1/1500 and 1/2000,
541 respectively. Each value represents the average of three independent experiments.

542 **Figure 4**. Standard curves for rMbST and bST obtained with the peptide-purified pAb
543 MU11.

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544 ***Analytica Chimica Acta***

545 **Highlights**

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548 - Production of polyclonal antibodies directed to recombinant methionyl bovine
549 somatotropin (rMbST)
550 - Multiple antigen peptide mimicking rMbST *N*-terminus used as immunogen
551 - Immunodiscrimination between native and recombinant bovine somatotropins by
552 ELISA

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Table 1. Pituitary and commercial recombinant bST specifications

Somatotropin	aa length	Molecular weight	Position 1	Position 1/2	Position 126/127
bST variant 1	191	21788 Da	Ala-[c]	-Phe-[d]	-Val-[e]
bST variant 2[a]	191	21802 Da	Ala-	-Phe-	-Leu-[f]
bST variant 3	190	21717 Da		Phe-	-Val-
bST variant 4	190	21731 Da		Phe-	-Leu-
rMbST (Posilac®)[b]	191	21851 Da	Met-[g]	-Phe-	-Leu-

[a] Majority isoform of pituitary bST. [b] Commercial recombinant bST most commonly used worldwide. [c] Alanine. [d] Phenylalanine. [e] Valine. [f] Leucine. [g] Methionine.

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Table 2. Polyclonal antibody production

Immunogen	Coupling chemistry	pAbs
rMbST–KLH[a]	active ester method (–CO_2H groups in rMbST; –NH_2 groups in KLH)	BC5, BC6, BC7, BC8
EP091213–KLH[b]	sulfo-SMCC method (–NH_2 groups in KLH; –SH group of Cys[d] in peptides)	M9, M10
MF-MAP-SMCC–KLH[c]		MU9, MU10[e], MU11, MU12

[a] Complete recombinant methionyl bST. [b] Synthetic rMbST *N*-terminus-mimicking linear peptide. [c] Synthetic rMbST *N*-terminus-mimicking multiple antigen peptide. [d] Cysteine. [e] This rabbit died during the immunization process.

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Table 3. Summary of the preliminary characterization of the pAbs by antigen-coated ELISA

Immunogen	pAb	Binding to rMbST	Binding to bST
rMbST–KLH	BC5	+++[a]	+++
	BC6	+++	+++
	BC7	+++	+++
	BC8	+++	+++
EP091213–KLH	**M9**	**+**	**+**
	M10	–	–
MF-MAP-SMCC–KLH	**MU9**	**+**	**+**
	MU11	**+**	**–**
	MU12	+++	+++

[a] Results corresponding to the analysis of the raw pAbs used without any further purification. Binding to coating antigens (rMbST and bST at 1 μg mL^{-1}) corresponding to a 1/500 dilution of the pAbs expressed as: (+++) strong (AU ≥ 2); (++) medium (1 ≤ AU < 2); (+) low (0.3 ≤ AU < 1); (–) negligible (AU < 0.3). Those pAbs providing a signal intensity for rMbST at least 0.2 UA above that observed towards bST have been highlighted in bold.

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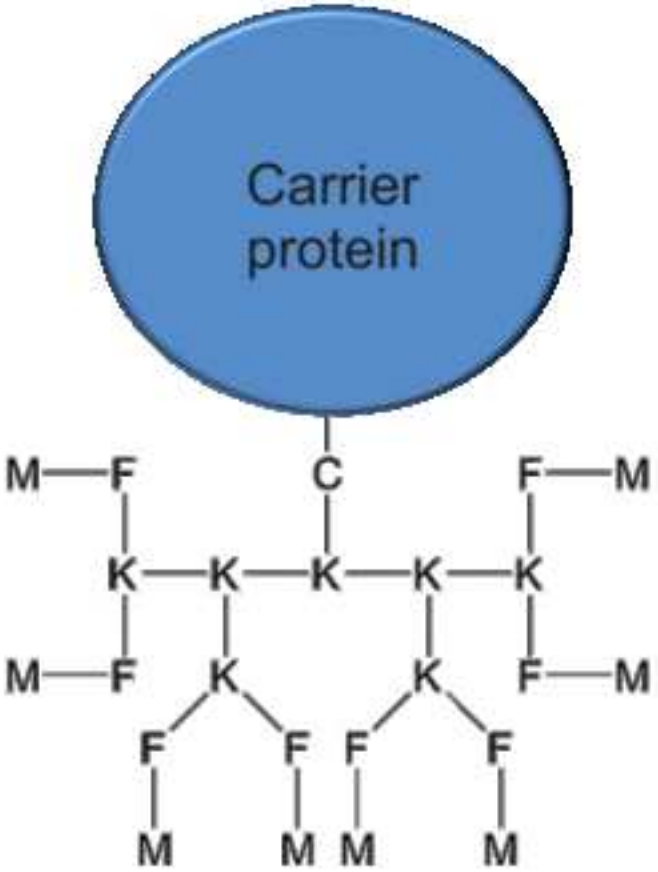
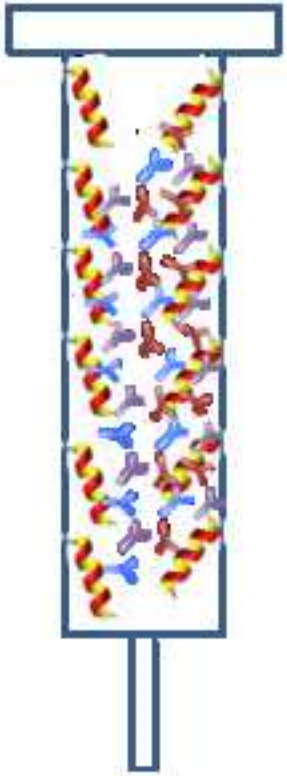
*Graphical Abstract

Immunodiscrimination between closely structurally related proteins

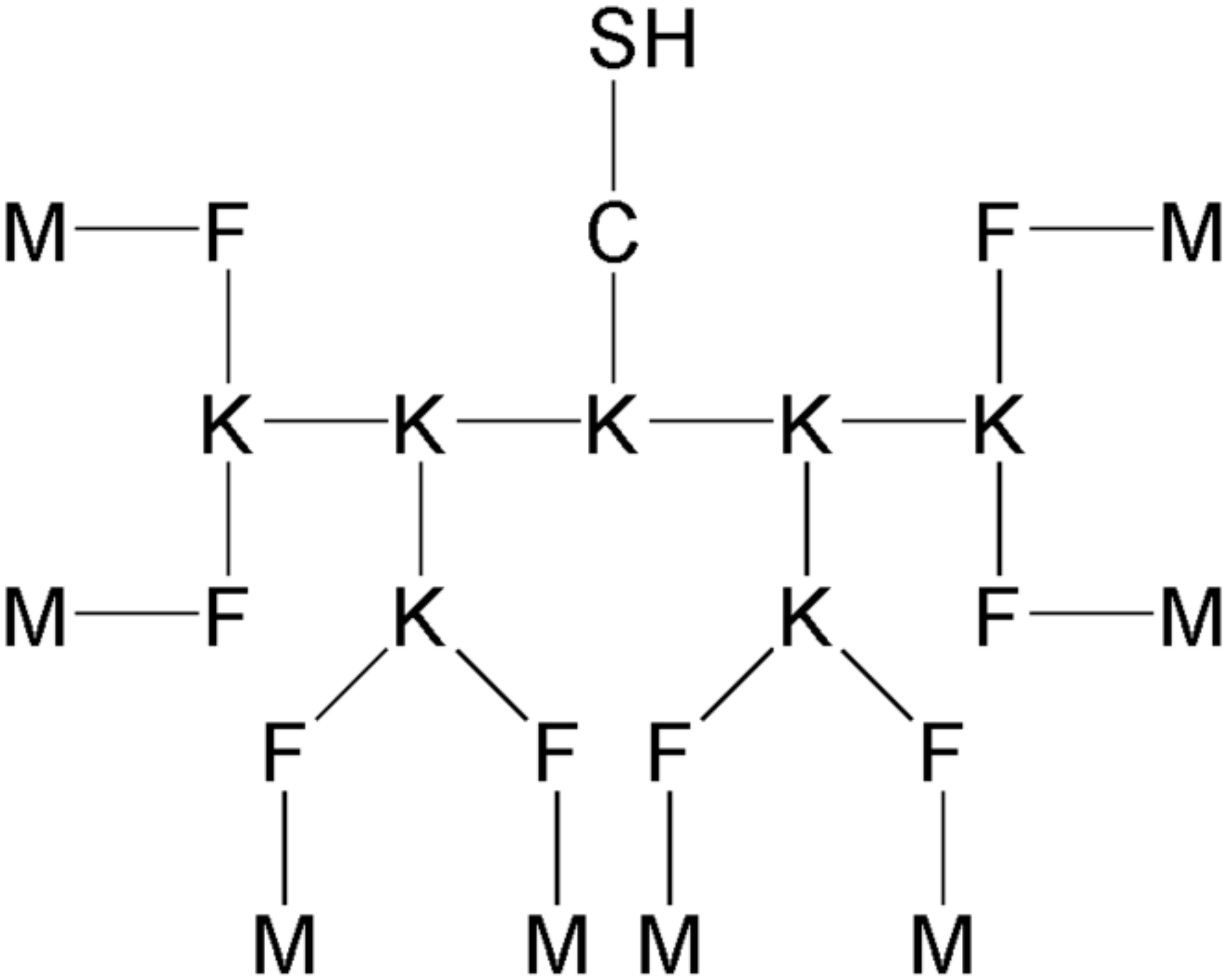




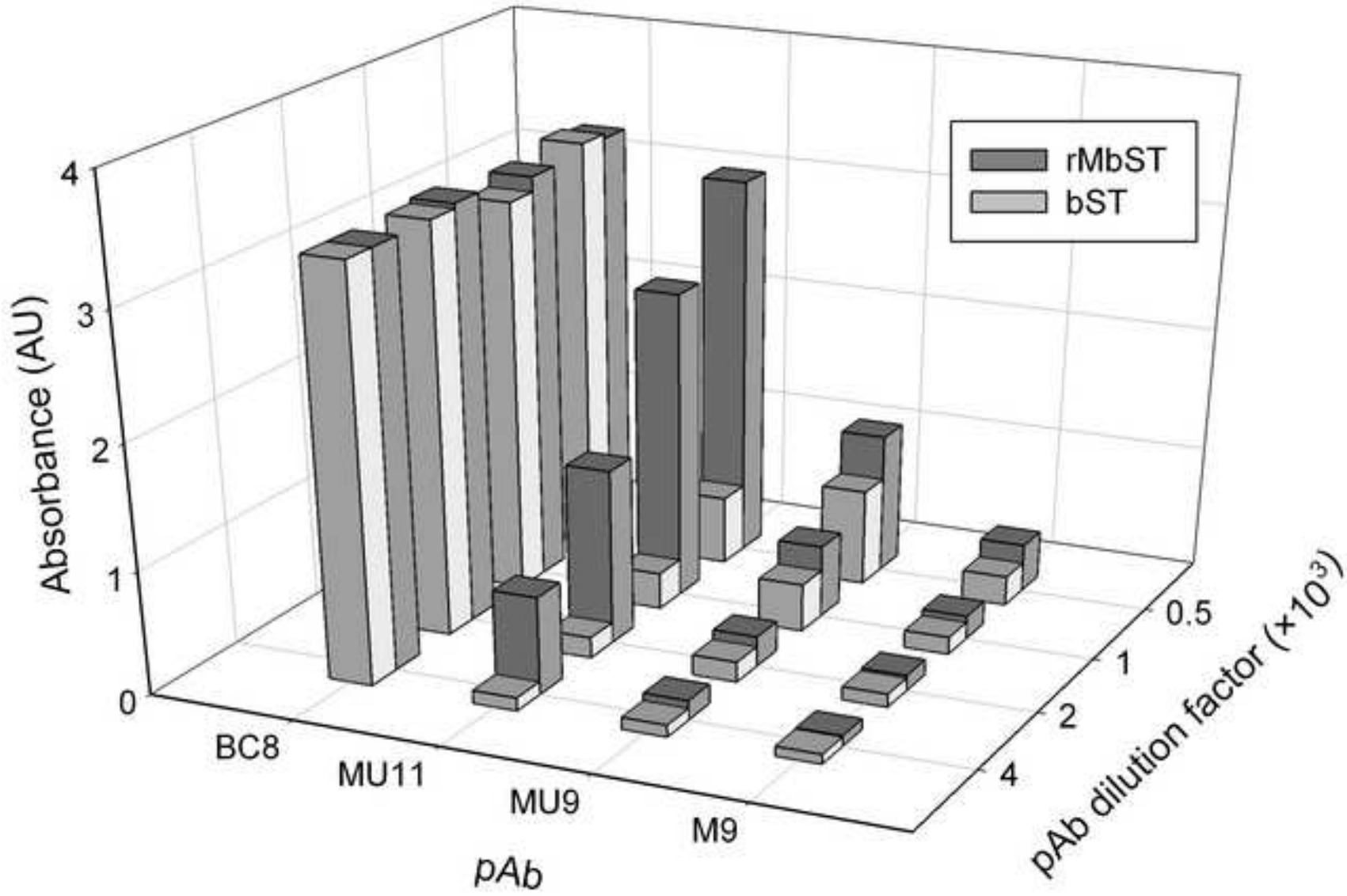
MAP-based immunogen

Monovalent peptide-based affinity purification of antibodies

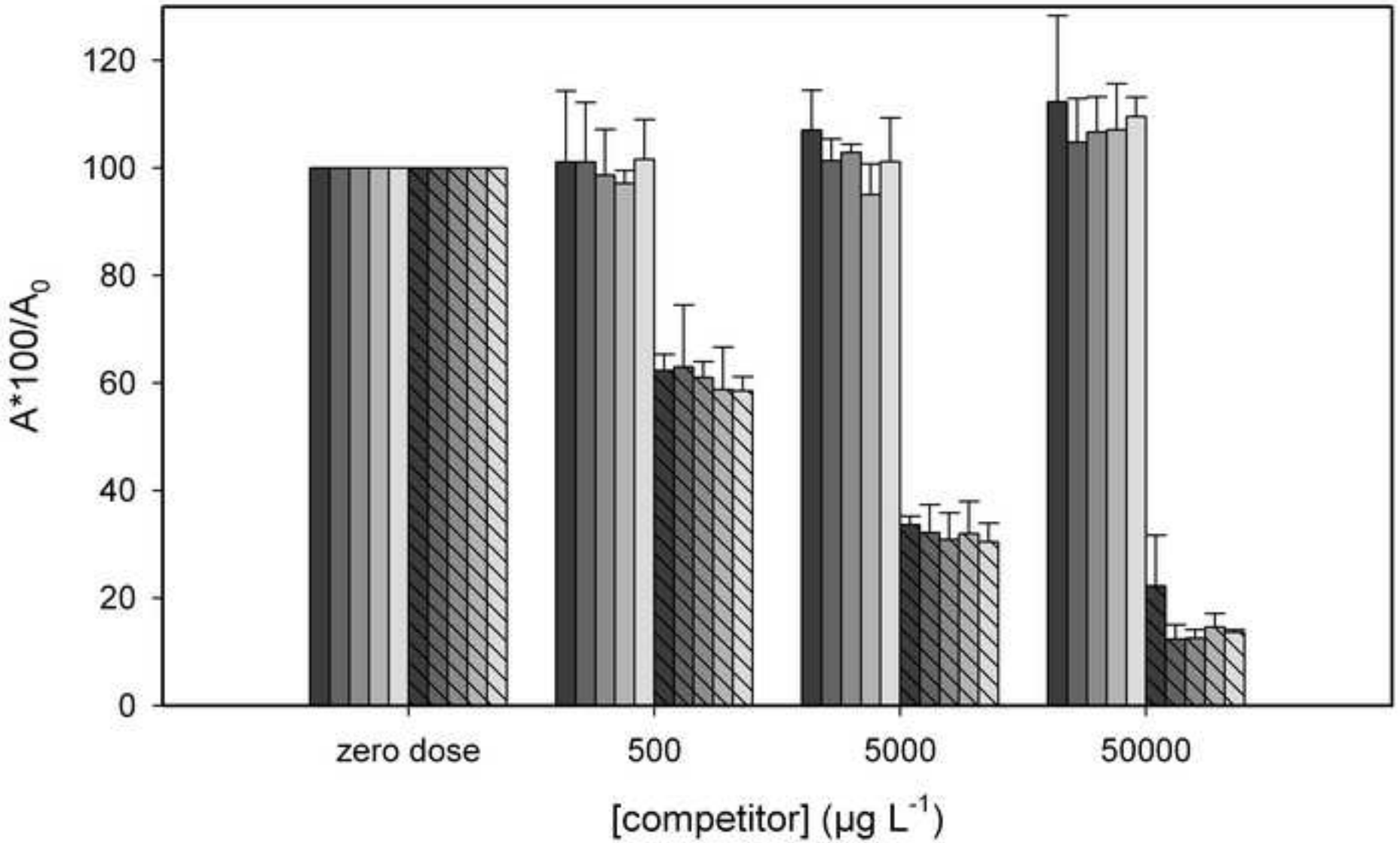
Figure



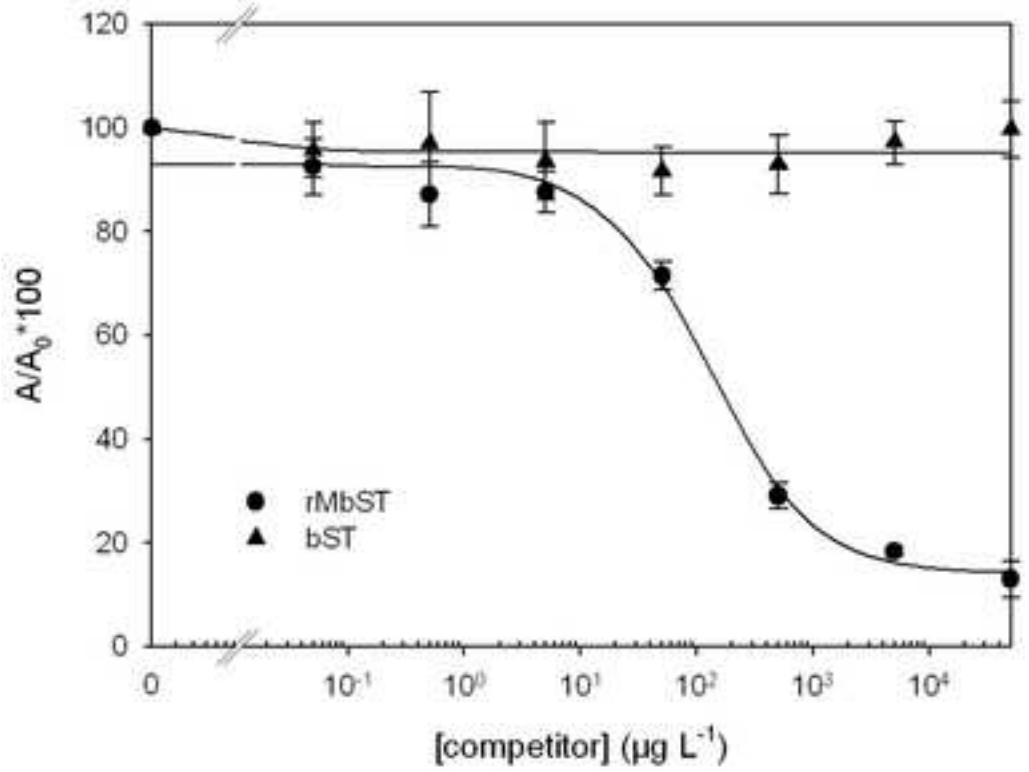
Figure


rMbST
bST
Absorbance (AU)
0
1
2
3
4
BC8
MU11
MU9
M9
pAb
0.5
1
2
4
pAb dilution factor (×10³)

Figure


120
100
80
60
40
20
0
A*100/A0
zero dose
500
5000
50000
[competitor] (µg L-1)

Figure



Assay conditions and analytical parameters of the rMbST standard curve	
antibody dilution	1/2000
coating (rMbST)	2 µg mL^{-1}
pre-incubation antibody + competitor	overnight (4 °C)
competition	30 minutes (37 °C)
A_{max}[a]	1.01 ± 0.18
slope	−0.96 ± 0.20
IC_{50} (µg L^{-1})	128 ± 16

[a] Each value represent the average ± sd of 4 independent experiments.